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A METHOD FOR THE SYNTHESIS OF RIBONUCLEOSIDE AND DEOXYRIBONUCLEOSIDE-5'-TRIPHOSPHATES

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SUMMARY

A method is described which permits the synthesis of ribonucleoside-, and deoxyribonucleoside-triphosphates in good yields and in amounts commensurate with their use in tracer experiments.

* A portion of the present work shall be presented as part of a research thesis in partial fulfillment of the requirements for the Degree of Doctor of Medicine according to the regulations of the Yale Medical School.

INTRODUCTION

We wish to present an enzymic method which permits the net synthesis of nucleoside triphosphates. This method uses enzyme systems isolated by simple fractionation procedures from *E. coli* B and from brewer's yeast. The specificity and the activity of the enzyme systems are wide enough to permit the synthesis of both ribonucleoside and deoxyribonucleoside triphosphates in relatively large amounts commensurate with the use of these compounds as substrates for the study of polymerization and other reactions.

METHODS AND RESULTS

E. coli *B Enzyme*

The growth medium contains (per l): Bactopeptone (Difco) (5 g), Bacto-yeast extract (Difco) (10 g), KH_2PO_4 (17 g), K_2HPO_4 (21.8 g), Antifoam AF (Dow Corning) (about 20 mg), and glucose (10.0 g). The glucose is autoclaved separately in 100 ml of water. The solutions, when prepared in lots of 1 l, are autoclaved for 15 min at a pressure of 15 lbs/sq. in; they are incubated for 10 min at the same pressure when prepared in 10-l lots. An initial 10 ml of bacterial culture is prepared by loop transfer from stock agar slants. After a 4- to 5-h period of incubation at 37°, the inoculum (10 ml) is poured under sterile conditions into 1 l of growth medium, which is stirred overnight at room temperature. This is then added to 10 l of growth medium, which is agitated by vigorous aeration, under sterile conditions, for 3 h at 37°. Then, when the bacteria are in the middle of the exponential phase of growth, the cells are collected by centrifugation at 1°. The cells are washed once by suspension in cold isotonic saline and centrifuged at 1° for 30 min at 30,000 $\times$ *g*. The yield is 100 $\pm$ 10 g (wet wt.) per 11 l of medium.

Disruption of the total mass of organisms is performed, in a Servall Omni-Mixer, by blending at top speed in the presence of 50 ml of 0.01 *M* potassium phosphate buffer (pH 7.4), and 250 g of glass beads* for 15 min at 0°. After the addition of another 250 ml of the cold phosphate buffer, the disruption in the Omni-Mixer is repeated for 15 min more. The mixture is then centrifuged for 30 min at 30,000 $\times$ *g* and the precipitate discarded. (In some experiments, in which the precipitate was re-extracted with 0.01 *M* potassium phosphate buffer, an appreciable amount of enzyme activity was recovered.) To 100 ml of the supernatant solution are added 4 ml of 1 *M* potassium phosphate buffer (pH 8.0), and the mixture is incubated for 1 h at 37°**. After centrifugation, ammonium sulfate is added to the supernatant solution to 55% saturation (35.2 g/100 ml) and the suspension is allowed to equilibrate for 30 min

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Abbreviations used in text: AMP, GMP, CMP, UMP represent the corresponding 5'-monophosphates of adenosine, guanosine, cytidine and uridine. dAMP, dGMP, dCMP, dTMP represent the 5'-monophosphates of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine. ATP, adenosine triphosphate; Tris, tris(hydroxymethyl) aminomethane; PRPP, 5'-phosphoribosyl-1'-pyrophosphate; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; CTP, cytidine triphosphate; UTP, uridine triphosphate.

* Glass type superbrite No. 100, 200 microns average diameter, Minnesota Mining Company, Reflective Products Division, St. Paul, Minnesota. We are indebted to Dr. S. OCHOA of New York University for information concerning this method. The glass beads are washed with water, ethyl alcohol and acetone before use.

** Personal communications from Dr. PAUL BERG, now of Stanford University.

in an ice bath. The suspension is then centrifuged at 30,000 × *g* for 30 min; it is necessary to centrifuge at high speeds in order to obtain a precipitate of minimum volume. The precipitate is dissolved in 20 ml of 0.1 *M* potassium phosphate buffer (pH 8.0); this results in a solution of pH 6.8–7.0. This solution is stirred in a water bath of 65° until its temperature reaches 50° and it is then transferred to a bath of 50° and kept there for 90 sec. The suspension is immediately cooled to 0° in an ice-bath, and is centrifuged at 1° for 10 min at 30,000 × *g*. The precipitate is discarded and the supernatant fluid is dialyzed overnight against two 4-l changes of 0.1 *M* potassium phosphate buffer (pH 7.4), or against two 4-l changes of 0.05 *M* potassium glycinate buffer (pH 8.1), when it is used for the synthesis of CTP from orotic acid.

Brewer's yeast enzyme

The method of preparation of this enzyme is identical to that used by LIEBERMAN *et al.*[1] for the preparation of orotidylic acid pyrophosphorylase through the alcohol precipitation step. The alcohol precipitate, obtained from an original extract of 30 g of brewer's yeast, is dissolved in 10 ml of 0.1 *M* potassium phosphate buffer (pH 7.4), and dialyzed overnight in the same way and with the same qualifications as described above for the *E. coli* enzyme.

Both enzyme preparations have been stored at —20° for periods up to 6 months without appreciable loss of activity. The precipitate which occurs upon freezing and thawing does not affect the total enzyme activity. Creatine kinase was prepared according to the short method of KUBY, NODA AND LARDY[2], but was not crystallized. The creatine phosphate and the nucleosides and nucleotides used in this study were commercial preparations. The PRPP used for the synthesis of UTP was prepared according to the method of KORNBERG *et al.*[3] and purified with charcoal[9]. The high content of inorganic phosphate in this preparation inhibits the synthesis of CTP[4]; for preparation of the latter from orotic acid the commercial magnesium salt of PRPP was employed.

Column chromatography

At the end of the incubation period, the incubation mixture is diluted 3- to 4-fold with water and and is transferred quantitatively to a column of Dowex-1-10X formate resin (20 cm × 1 cm); after addition of the sample the column is washed with 20 to 30 ml of water. This procedure permits the removal of the protein without acid deproteinization and avoids losses of triphosphates which might occur with the latter method. It also avoids overloading of the column with large amounts of anions which might accumulate if acid deproteinization were used.

The products of the enzymic reaction are separated by column chromatography using the ammonium formate system of pH 4.25 which was previously described[5]. This system, in addition to the fine separation of the deoxynucleoside-5'-monophosphates, affords excellent separation of the nucleoside mono-, di- and triphosphates involved in these studies. The peaks tend to be narrow and well-defined and emerge more rapidly than when ammonium formate solutions of pH 5.0[6] are employed. With the ammonium formate system of pH 4.25, GTP appears immediately after ATP. When the incubation mixtures contain large amounts of salts, elution of all nucleoside triphosphates may be accomplished best with 1 *M* ammonium formate in the reservoir. When small amounts of salts are present, it is necessary to change to 2.0 *M*

ammonium formate of pH 4.25 after approximately 100 fractions of 5 to 6 ml each have been eluted. Under the latter conditions, elution of GTP is usually completed when 150 to 170 fractions have been collected.

Isolation of nucleoside triphosphates

The fractions corresponding to the individual nucleoside triphosphates are pooled, adjusted to pH 5 to 7, diluted 5- to 10-fold and passed through a column of Dowex-1-chloride (1 cm × 1 cm). The column is washed with 10 ml of water and with 6 ml of 0.1 *M* NaCl to remove excess formate and other contaminants and is then eluted with 3 *M* NaCl until the nucleoside triphosphates are removed. After dilution of the eluate to 12 ml, in an ice-bath, the pH is adjusted to 8.5 and 1.0 ml of 1.3 *M* $Ba(ClO_4)_2$ is added. (The latter is prepared by neutralization of $HClO_4$ with $Ba(OH)_2$ to pH 8.5). To this cold solution, 2.5 volumes of cold 95 % ethanol are added and the barium salts of the nucleoside triphosphates are allowed to precipitate over a period of 2 to 3 h. The barium salts are then collected, washed first with alcohol and then with ether, and dried.

The sodium salts of the nucleoside triphosphates may be obtained by dissolving the corresponding barium salts in 0.1 *N* HCl, precipitating the barium as $BaSO_4$ with sodium sulfate, and neutralizing the supernatant solution. An alternative procedure is to stir a suspension of the barium nucleoside triphosphates with about 1 ml of Dowex-50-H^+-8X or Dowex-50-Na^+-8X for 15 min in an ice-bath. (Before using this resin it should be thoroughly washed with water until the pH of the eluate is about 5.) The supernatant solution is then collected, the resin washed with water, and the combined solutions neutralized with the appropriate base. Occasionally the resin eluate may contain traces of barium, which can be removed as barium sulfate. Yields of the sodium nucleoside triphosphates during the isolation procedure, from the column fractions to the sodium salts, are generally from 85 to 90 %.

TABLE I

PRODUCTS OF ENZYMIC REACTION

Substrate	*Enzyme*	*% of Substrate*			*Acid-Labile P:Total P*
		Mono-P	*Di-P*	*Tri-P*	
dAMP*	*E. coli* B			58	2.38:3.17
dCMP	*E. coli* B	12–18	9	58–68	2.01:3.22
dTMP	*E. coli* B	77	—	22	—
dGMP	*E. coli* B	—	9	75	2.18:2.96
AMP	*E. coli* B	2	9	89	2.15:3.15
GMP	*E. coli* B	3	6	91	2.18:3.13
UMP	*E. coli* B	3	3	90–95	2.02:3.20
CMP	*E. coli* B	20–41	4–7	51–70	2.02:3.24
Guanosine	*E. coli* B	4	8	88	2.15:3.05
Orotic acid	Brewers yeast	UMP	UDP	UTP	2.03:3.17
		5	5	90	
Orotic acid	Brewers yeast plus *E. coli* B	UMP	UDP	UTP	—
		—	—	14	
		CMP	CDP	CTP	2.02:3.24
		—	—	61	

* The dATP fraction is not separated from ATP. The yields of dATP have been corrected for the ATP added. Should selective removal of ATP in a mixture of ATP and dATP be desired, this may be accomplished by the method of LEHMAN *et al.*[12], or by that of KLENOW AND LICHTLER[15].

Identification of the final products was based on the ultraviolet spectral data[7,8], position of the eluted peaks on chromatograms (as compared with those of known samples), and by determination of the content of acid-labile and total phosphate. The incubation conditions, the yields of mono-, di- and triphosphates, and the phosphate analyses of the triphosphates synthesized, are summarized in Table I.

Incubation conditions

Substrates: dCMP, dTMP, UMP, CMP, AMP. Total volume, 5.0 ml, contained: substrate 15.9 to 19.9 μmoles, $MgCl_2$ 67 μmoles, ATP 6.4 μmoles, creatine phosphate 77.1 μmoles, creatine kinase 400 μg, Tris buffer (pH 7.4) 200 μmoles, 1.0 ml of enzyme. Incubation: 20 min at 38°.

Substrates: dAMP, dGMP. Total volume, 5.0 ml, contained: Substrate 15.9 to 17.1 μmoles, $MgCl_2$ 67 μmoles, ATP 1.6 μmoles, creatine phosphate 123.3 μmoles, creatine kinase 400 μg, Tris buffer (pH 7.4) 200 μmoles and 1.0 ml of enzyme. Incubation: 40 min at 38°.

Substrates: guanosine, GMP. Incubation conditions were the same as for dAMP and dGMP, except that potassium phosphate buffer (pH 7.4) was used in place of Tris buffer.

Substrate: orotic acid

(1) CTP Synthesis. Total volume, 7.6 ml, contained: orotic acid 10 μmoles, MgPRPP 14 μmoles, $MgCl_2$ 50 μmoles, NH_4Cl 50 μmoles, ATP 6.4 μmoles, creatine phosphate 77.1 μmoles, creatine kinase 500 μg, potassium glycinate (pH 8.1) 800 μmoles and 2.0 ml each of brewer's yeast enzyme and *E. coli* enzyme (both dialyzed against 0.05 *M* potassium glycinate (pH 8.1). Incubation: 60 min at 38° (after 30 min of incubation an additional 0.4 ml of solution was added: this contained ATP 3.1 μmoles, creatine phosphate 30.8 μmoles and creatine kinase 200 μg).

(2) UTP Synthesis. For the synthesis of UTP, the NH_4Cl and *E. coli* enzyme were omitted and PRPP, prepared as described[3,9], was used in place of the commercial MgPRPP.

DISCUSSION

The objective of this study was to devise a simple method, which would supplement the methods available in the literature[5,9-13] for the synthesis of radioactive ribonucleoside and deoxyribonucleoside triphosphates from starting materials which are more easily obtainable in radioactive form. The [^{14}C]ribonucleoside- and [^{14}C]-deoxyribonucleoside-5′-monophosphates are readily prepared by the action of venom diesterase[14] on the respective [^{14}C]nucleic acids of *E. coli* grown on $^{14}CO_2$. RNA and DNA, uniformly labeled with ^{14}C, are also commercially available. [^{14}C]guanosine may be obtained either commercially, or by the action of intestinal phosphatase on [^{14}C]guanylic acid (2′,3′); the latter may be prepared by alkaline hydrolysis of [^{14}C]-RNA. The broad specificity of the enzyme preparation from *E. coli* permits the conversion of guanosine and the nucleoside-5′-monophosphates to the corresponding triphosphates in high yield; however, dTMP is more slowly phosphorylated. Nevertheless, the low phosphatase content of the preparation permits the use of prolonged incubation periods when larger amounts of product are desired.

Uridine, cytidine and adenosine cannot serve adequately as precursors of the corresponding triphosphates with the *E. coli* enzyme, since the enzyme contains powerful uridine nucleosidase and cytidine and adenosine deaminase activities. Thymidine is extensively phosphorolyzed by this enzyme. An alternative approach to the synthesis of labeled pyrimidine ribonucleoside-5′-triphosphates involves the use of [^{14}C]orotic acid as substrate with the brewer's yeast enzyme, either alone for the preparation of UTP, or in combination with the *E. coli* enzyme for the preparation of CTP. The *E. coli* enzyme contains the UTP aminase described by LIEBERMAN[4] and, when it is coupled with the brewer's yeast system in the presence of NH_4^+, orotic acid is extensively converted to CTP. Since phosphate and tris are inhibitory to the amination of UTP[4], the preparation of CTP by this method is performed with enzymes dialyzed against 0.05 *M* potassium glycinate (pH 8.1).

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